Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 6953–6958, June 1999
Medical Sciences

Phage-display library selection of high-affinity human single-chain antibodies to tumor-associated carbohydrate antigens sialyl Lewis^x and Lewis^x

SHENLAN MAO, CHANGSHOU GAO, CHIH-HUNG L. LO, PETER WIRSCHING, CHI-HUEY WONG, AND KIM D. JANDA*

Departments of Chemistry and Molecular Biology, The Scripps Research Institute and the Skaggs Institute for Chemical Biology, 10550 North Torrey Pines Road, La Jolla, CA 92037

Communicated by Julius Rebek, Jr., The Scripps Research Institute, La Jolla, CA, April 2, 1999 (received for review January 20, 1999)

ABSTRACT **mAbs against tumor-associated carbohydrate antigens have the potential to play a prominent role in cancer immunotherapy. However, it has not been possible to fully exploit the clinical utility of such antibodies primarily, because those of adequate affinity could be derived only from murine sources. To address this problem, we prepared a single-chain Fv (scFv) antibody library from the peripheral blood lymphocytes of 20 patients with various cancer diseases. Completely human high-affinity scFv antibodies were then selected by using synthetic sialyl Lewis^x and Lewis^x BSA conjugates. These human scFv antibodies were specific for sialyl Lewis^x and Lewis^x, as demonstrated by ELISA, BIAcore, and flow cytometry binding to the cell surface of pancreatic adenocarcinoma cells. Nucleotide sequencing revealed that at least four unique scFv genes were obtained. The K_d values ranged from 1.1 to 6.2 $\times$ 10^{-7} M that were comparable to the affinities of mAbs derived from the secondary immune response. These antibodies could be valuable reagents for probing the structure and function of carbohydrate antigens and in the treatment of human tumor diseases.**

Essentially all cancer cells are characterized by aberrations in the structure and function of the surface membrane. Over the past three decades, substantial evidence has accumulated that now concretely associates major changes in ganglioside and/or glycoprotein expression and composition with oncogenic transformation (1–5). Significantly, these changes were found to occur in a wide range of carcinomas and to be correlated with tumor progression, metastasis, and patient survival rates (6, 7). The development of mAbs against carbohydrate antigens has generated considerable interest, given the potential of these mAbs for the immunodiagnosis and immunotherapy of cancer (8–12).

To date, monoclonal IgGs against carbohydrate antigens have been murine antibodies obtained by using conventional hybridoma technology and through selection from recombinant antibody libraries (11, 13–15). However, results in human testing experiments have been generally disappointing because of the invariable induction of a human antimurine antibody response (16–18) that enhances the clearance of the murine antibodies from the circulation and also causes side effects that hamper high-dosing, repetitive or long-term therapeutic paradigms. Methods designed to obtain "humanized" versions of murine mAbs via complementarity-determining region grafting or antibody veneering are not versatile and in addition produce antibodies that still retain immunogenicity (19–21). Hence, although recent chimeric and humanized constructs of promising mAbs might indeed provide some improvements, practical results remain to be resolved in clinical trials (22, 23).

In recent years, phage-display technology has allowed one to generate completely human mAbs from both immune and nonimmune sources (24–26). Significantly, formatting of the libraries for single-chain antibody representation has provided single-chain Fv antibody (scFv) fragments expected to have improved tumor penetration and pharmacokinetic properties (27, 28). Herein, we have exploited a phage-display library derived from the blood of cancer patients to select and isolate high-affinity scFv antibodies specific for the carbohydrate antigens sialyl Lewis^x (sLe^x) and Lewis^x (Le^x).

MATERIALS AND METHODS

Preparation of cDNA Templates. Blood samples (20 ml) were donated by 20 different patients with various cancer diseases. The cells from each individual were pelleted and the peripheral blood lymphocytes (PBLs) were separated on Histopaque gradients (Sigma). Total RNA was prepared individually from the 20 samples of PBLs by using RNA purification kits from Stratagene. First-strand cDNA was also individually synthesized from total RNA by using cDNA synthesis kits (Amersham Pharmacia) with random hexamers as primers.

Construction of scFv Antibody Library. The primers were based on those published previously and the most recent gene segments entered in the V-Base sequence directory (29, 30). For human heavy-chain (V_H) gene repertoires, 12 separate PCRs were set up by using one of 12 different HVH back primers in each reaction along with an equimolar mixture of HJH forward primers. For the kappa and lambda light-chain (V_L) genes, the same approach was used with 13 separate reactions defined by individual HVκ/HVλ back primers and a mixture of HJκ/HJλ forward primers. PCRs were performed in 100-ml volumes containing 2 μl of cDNA reaction mixture, 2 μM primer solutions, 200 μM dNTPs, 5% DMSO, and 10 μl *Taq* polymerase reaction buffer. After 5 min of denaturation at 94°C, 5 units of *Taq* polymerase was added, followed by 30 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C, and at the end of cycling an incubation of 10 min at 72°C. After PCR, the various reactions afforded V_H, V_κ, and V_λ subpools from each of the 20 different patients that were mixed to give three final V_H, V_κ, and V_λ pools ready for purification and assembly. The amplified V_H and V_L genes were gel-purified on agarose. The scFv genes were generated by assembly of V_H, the $(Gly_4Ser)_3$ linker, and V_L fragments. First, approximately 20 ng each of V_H, linker, and V_L were assembled by PCR without primers in which the short regions of complementarity built into the ends of the linker drive hybridization of the various fragments. An initial denaturation step (5 min, 94°C) was followed by five cycles of 1 min at 94°C, 1 min at 60°C, and 1.5

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

Abbreviations: sLe^x , sialyl Lewis^x; Le^x, Lewis^x; scFv, single-chain Fv antibody; SB, super broth; PBL, peripheral blood lymphocyte; FACS, fluorescence-activated cell sorting.

*To whom reprint requests should be addressed. e-mail: kdjanda@scripps.edu.

min at 72°C in the absence of primers. After adding the outer primers HVH (*Sfi*I) and HJL (*Sfi*I), 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1.5 min at 72°C were performed. The scFv genes were digested with *Sfi*I, agarose gel-purified, and ligated into the phage-display vector pCGMT (31) that had been cut with the same restriction enzyme. The ligated products were electroporated into *Escherichia coli* XL1-Blue cells to yield approximately 2×10^8 clones. After electroporation, cells were plated on super broth (SB) medium (containing 1% glucose, 50 μg/ml carbenicillin, and 40 μg/ml tetracycline) in 10 dishes (530 cm^2) (Nunc) and incubated overnight at 30°C. The clones were scraped off the plates into 50 ml SB medium with 10% glycerol and subsequently stored at −70°C.

Rescue of scFv-Displaying Phage. To rescue scFv-displaying phage, 200 ml SB medium (containing 2% glucose, 50 μg carbenicillin, 10 μg tetracycline) was inoculated with approximately 2×10^9 cells from the glycerol stock library. The culture was then shaken at 37°C until OD_{600} reached about 0.5, and 10^{12} colony-forming unit of VCSM13 (Stratagene) and 200 μl of 1 M isopropyl β-D-thiogalactopyranoside were added. After 30-min incubation at room temperature, the culture was diluted in 400 ml SB (containing carbenicillin and tetracycline) and grown at 30°C. After 2 h, 70 μg/ml kanamycin was added and the culture was allowed to grow overnight. Phage particles were purified and concentrated by polyethylene glycol/NaCl precipitations.

Selection of Phage Antibodies. The library was subjected to four rounds of panning. Immunotubes (Maxisorb, Nunc) were coated overnight at 4°C with 1 ml of 10 μg/ml sLe^x-BSA (Oxford GlycoSystems, Bedford, MA) in PBS (10 mM phosphate/150 mM NaCl, pH 7.4) except for the last round of panning when the tube was coated with only 1 μg/ml sLe^x-BSA to enrich the higher affinity phage. After blocking with 4% skimmed milk powder in PBS for 1 h at 37°C, 10^{12} colony-forming unit phage particles in 2 ml PBS containing 1% milk and 3% BSA were added and incubated for 2 h with rocking at 37°C. The tube was washed with PBS/0.1% Tween and PBS (twice for round 1, five times for round 2, and 20 times for subsequent rounds). Bound phage were eluted from the tube with 1 ml of decreasing concentrations (100 μM, 10 μM, 1 μM, and 0.01 μM in PBS) of sLe^x (Oxford GlycoSystems). Eluted phage were amplified by infection of fresh *E. coli* XL-1-Blue cells and the phage rescued as outlined above.

ELISA. Specificity of individual phage scFv and soluble scFv were assessed by ELISA. The sLe^x-BSA or Le^x-BSA were applied to a microtiter plate at a concentration of 5 μg/ml in PBS at 4°C overnight. After being washed three times with water, the wells were blocked with Blotto (4% skimmed milk in PBS) for 1 h at 37°C. Then, 25 μl/well scFv phage or soluble scFv was added and incubated for 1 h at 37°C. After washing, for phage ELISA, 25 μl of horseradish peroxidase (HRP)-conjugated mouse anti-M13 (Amersham Pharmacia) in Blotto with 1:1,000 dilution was added for 30 min at 37°C. For ELISA using soluble scFv, mouse anti-Flag mAb was added for 30 min at 37°C, and, after washing, HRP-conjugated goat anti-mouse Ig was added for 30 min. For detection, 50 μl/well of TMB substrate (Pierce) was used and the absorbance read at 450 nm.

scFv Purification. The scFv gene fragments were subcloned into pETFlag (derived from pET-15b, Novagen) and transformed into *E. coli* B834(DE3) cells (Novagen). The scFv expression was induced by growth in SB containing 0.5 mM isopropyl-D-thiogalactoside overnight at 30°C. The Flag-tagged scFvs were purified on anti-Flag M2 affinity agarose (Sigma) from the periplasmic extracts and media. The yield of purified scFv in pETFlag varied between 0.1 and 1 mg/liter. Purified monomeric scFv was prepared by Sephacryl-100 chromatography on FPLC (Amersham Pharmacia) by using PBS buffer.

Surface Plasmon Resonance (SPR) Analysis. Binding kinetics were determined by SPR by using a BIAcore biosensor system (Amersham Pharmacia Biosensor) (32, 33). The sLe^x-sp-biotin (GlycoTech, Rockville, MD) was immobilized on SA sensor chips by using conjugate concentrations of 5 μg/ml in PBS and contact times of 8 min at a flow rate of 5 μl/min, according to the instructions of the manufacturer. The Le^x-BSA conjugate was immobilized on CM5 senor chips in 10 mM formate (pH 3.0) by using the standard amine immobilization procedure. Conjugate concentrations of 10 μg/ml and contact times of 2 min at a flow rate of 10 μl/min gave approximately 250 relative units. All measurements were conducted in HBS buffer (Amersham Pharmacia Biosensor) with a flow rate of 10 μl/min. In all cases, 20 μl of different concentrations of scFv antibodies was diluted in HBS buffer and injected into the BIAcore. After each measurement, the chip surface was regenerated with 10 μl of 1 mM HCl.

BIAcore Two-Site Binding Assay. Purified scFvs S7, S8, and S10 were individually immobilized on CM5 sensor chips in 5 mM maleate buffer, pH 6, by using the standard amine immobilization procedure. Antibody concentrations of 10 μg/ml and contact times of 2 min at a flow rate of 10 μl/min gave approximately 500 response units. All measurements were conducted in HBS buffer with a flow rate of 10 μl/min. A 5-μl volume of sLe^x (Glycotech) at 50 μg/ml in HBS buffer was injected, followed by 20 μl of a second scFv (50 μg/ml in HBS), different from the one immobilized. After each measurement, the chip surface was regenerated with 10 μl of 10 mM HCl. A negative control was performed by directly injecting the second antibody without the injection of sLe^x.

Sequence Analysis of scFvs. Nucleic acid sequencing of selected clones was carried out on a 373-A automated DNA sequencer (Applied Biosystems). All sequences were searched in the Kabat database (http://www.ncbi.nlm.nih.gov) to compare them with previously sequenced V_H and V_L chains. The International Immunogenetics database (http://www.Genetik.uni-Koeln.de/dnaplot) was used to propose correlations of scFvs with potential germline gene sequences and assess V-segment usage. The MACVECTOR (Eastman Kodak) sequence analysis software was used for alignment and sequence manipulations.

Fluorescence-Activated Cell Sorting (FACS) Analysis. Specific binding of the scFvs directed to cell-surface sLe^x and Le^x was measured by FACScan (Becton Dickinson). About 300,000 human pancreatic adenocarcinoma cells (cell line SW1990; American Type Culture Collection) (34) were used in each experiment. The tumor cells in PBS containing 1% BSA were incubated on ice for 1 h with the scFvs (10 μg/ml) (no scFv antibody was used for the negative control), 30 min with 10 μg/ml anti-Flag M2 mAb (Sigma), and 30 min with fluorescein-conjugated $F(ab')_2$ goat anti-mouse IgG (Pierce) diluted 1/50. Two washes in PBS/1% BSA followed each step. For each sample, 10,000 cells were analyzed with the CELL QUEST program (Becton Dickinson).

RESULTS

Library Generation and Panning. The cDNA was generated by reverse transcription from RNA isolated from PBLs of 20 cancer patients. The cancer phenotypes and the corresponding numbers of patients were: breast (5), multiple myeloma (3), testicular (2), colon (2), non-Hodgkin's lymphoma (2), lung (1), stomach (1), malignant paraganglioma (1), cholangiocarcinoma (1), hairy-cell leukemia (1), and acute leukemia (1).

The gene fragments corresponding to the variable regions of the V_H and V_L of the repertoire of antibody genes were amplified by PCR. For more diverse repertoires, we used V_H, λ, and κ gene family specific primers individually for the amplification. The gene fragments were joined to form a series of scFv gene sequences that were ligated into the phagemid vector pCGMT. To examine the integrity of the library, 20 clones were picked at random, and all were found to contain

Table 1. Analysis of V_H and V_L gene usage in the scFv antibodies

Clone	V_H gene		V_L gene	
	V_H family	V_H segment	V_L family	V_L segment
S6	VH1	DP-75	Vλ5	5C.400B5
S7	VH3	DP-47	Vλ5	5C.400B5
S8	VH1	DP-75	Vλ5	5C.400B5
S10	VH1	DP-75	Vλ5	5C.400B5

scFv nucleotide sequences were analyzed by searching the IMGT database using the DNAPLOT software. The complete protein sequences are shown in Table 2.

inserts of the appropriate size having diverse DNA sequences. The panning was performed for a total of four rounds with an enrichment of clone number from 4×10^5 in the first panning to 8×10^9 in the fourth panning. Recombinant phage expressing a library of scFv antibodies on their surface were produced by helper phage rescue and selectively enriched by panning against immobilized sLe^x-BSA conjugate. The phage were pooled after each round of panning and tested for their ability to bind to sLe^x-BSA by ELISA. The pool of phage rescued from the library before panning showed no binding to sLe^x-BSA. However, after four rounds of selection, phage showed greatly enhanced specific binding to sLe^x-BSA (data not shown). The specificity of the interaction with sLe^x-BSA was indicated by the absence of binding of the phage population to BSA itself.

The pool of phage selected by four rounds of panning was used to infect XL1-Blue cells, and subsequent plating on agar containing carbenicillin allowed individual clones harboring phagemid to grow. To verify that individual phage clones could bind to sLe^x-BSA, phage were rescued from 94 randomly selected clones and tested for binding to sLe^x-BSA by ELISA. Of the 94 clones, 72 exhibited binding to immobilized sLe^x-BSA. In contrast, of 94 random clones selected from the unpanned library, none showed significant binding activity.

To produce soluble scFvs, *E. coli* HB2151 cells (Amersham Pharmacia Biotech) were infected with phage obtained after four rounds of panning. Recognition of the amber stop codon between the scFv and M13 gene III protein resulted in the production of soluble scFv by this strain. The soluble scFvs were directed to the periplasmic space by a pelB leader sequence. Incorporation of a small peptide epitope (Flag-tag) at the carboxyl-terminal end of the scFv allowed detection with an anti-Flag M2 antibody. On induction with isopropyl D-thiogalactoside, an immunoreactive band with an apparent molecular mass of about 30 kDa was detected in the periplasmic extracts from *E. coli* infected with phage. The periplasmic extract prepared from HB2151 cells infected with phage contained functional scFv that bound to sLe^x-BSA and could discriminate between sLe^x-BSA and BSA even at a 1:2,000 dilution of the extract.

Sequences of scFv Clones S6, S7, S8, and S10. The nucleotide sequences encoding the V_H and V_L chains were determined. Analysis of 24 different clones that demonstrated strong reactivity to sLe^x-BSA by ELISA revealed four se-

Table 2. Complete protein sequences

Clone	Heavy chain	Light chain
FR1		
S6	QVQLV**E**SGAEVKKPGASVKVSCKASGYTFT	QAVLTQPSSLSASPGAS**V**SLTC
S7	**Q**VQL**Q**ESGGGLVQPGGSLRLSC**V**ASGFTF**R**	QAVLTQPSSLSASPGASASLTC
S8	**H**VQLVQSGAEVKKPGASVKVSCKASGYTFT	QAVLTQPSSLSASPGASVSLTC
S10	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	QAVLTQPSSLSASPGAS**V**SL**I**C
CDR1		
S6	GYYMH	TLRSGINVGA**Y**RIY
S7	SYAMS	TLRS**D**INV**RS**YRIY
S8	GYYMH	TLRSGINVG**A**YRIY
S10	GYYMH	TLRSGINVG**A**YRIY
FR2		
S6	WVRQAPGQGLEWMG	WYQQKPGSPPQ**F**LLR
S7	WVRQAPGKGLEWVS	WYQQKPGSPPQYLLR
S8	WVRQAPGQGLEWMG	WYQQKPGSPPQ**F**LLR
S10	WVRQAPGQGLEWMG	WYQQKPGSPPQ**F**LLR
CDR2		
S6	WINPNSGGTNYAQKFQG	YKSDSDKQQGS
S7	**T**ISGSGGSTYYADSVKG	YKSDSDK**H**QGS
S8	WINPNSGGTNYAQKFQG	YKSDSDKQQGS
S10	WINPN**T**GGT**K**Y**T**QKFQG	YKSDSDKQQGS
FR3		
S6	RVTMTRDTSISTAYMELSRLRSDDTAVYYCRQ	GVPSRFSGS**R**DASANAGILLISGLQSEDEADYYC
S7	RFTISRDN**A**KN**S**LYLQMNSLRAEDTAVYYC**GR**	GVPSRFSGS**R**DASANAGIL**V**ISGLQSEDEADYYC
S8	RVTMTRDTSISTAYMELSRLRSDDTAVYYCAR	GVPSRFSGS**R**DASANAGILLISGL**R**SEDEADYYC
S10	RVTMTRDTSISTAYMELSRLRSDDTAVYYCAR	GVPSRFSGS**R**DASANAGILLISGL**R**SEDEADYYC
CDR3		
S6	GGYGPRGYFDI	AIWHSSAWV
S7	SVVGY	AIWHSSTWV
S8	AGRFGELY	AIWHSSAWV
S10	VKSGAFDI	AIWHSSAWV
FR4		
S6	WGQRDHGHRLLS	FGGGTQLTVLG
S7	SQGTLVTVSS	FGGGTKLTVLG
S8	WGQGTLVTVSS	FGGGTQLTVLG
S10	WGQGTMVTVSS	FGGGTKLTVLG

The boldface letters (from FR1 through FR3) indicate the amino acid differences between V_H and V_L domains of scFv clones and the closest germ-line V segments. FR, framework region; CDR, complementarity-determining region.

Table 3. Kinetics and thermodynamics of binding of sLe^x and Le^x conjugates to scFvs

	sLe^x-biotin			Le^x-BSA		
scFv	k_{on} ($\times 10^4$ M^{-1} s^{-1})	k_{off} ($\times 10^{-3}$ s^{-1})	K_d ($\times 10^{-7}$ M)	k_{on} ($\times 10^4$ M^{-1} s^{-1})	k_{off} ($\times 10^{-3}$ s^{-1})	K_d ($\times 10^{-7}$ M)
S6	4.3	4.7	1.1	1.3	8.3	6.2
S7	1.1	5.3	4.9	2.6	7.3	2.9
S8	5.1	9.1	1.8	2.3	6.5	2.9
S10	1.2	6.7	5.7	2.1	4.6	2.2

The kinetic constants of scFvs were measured using the BIAcore biosensor: k_{on}, association rate constant; k_{off}, dissociation rate constant; K_d, calculated ($K_d = k_{off}/k_{on}$).

quences (S6, S7, S8, and S10) that differed in V_H and V_L (Tables 1 and 2). As expected, the majority of the diversity was contained within the complementarity-determining region 3 of the V_H chain that varied both in sequence and in length. The V_H genes of S6, S8, and S10 scFvs belonged to the same VH1 family, derived from the same germ-line segment DP75, and showed a different number and position of mutations from the germ line. The V_H gene of the S7 antibody belonged to the VH3 family and was derived from the germline segment DP47. The light chains of all four antibodies were of the Vλ-5 family and consisted of segment 5C.400B5. As reported by others, antibodies derived from the same library frequently used the same light-chain segments (26). The DNA sequences from both the V_H and V_L were searched against the Kabat database and all were shown to be unique sequences. Because only a small number of clones from the selection were analyzed, screening of more clones would likely yield additional unique antibodies.

Binding Specificity and Affinity of Selected scFv Antibodies. To analyze binding specificity and affinity, the genes of four scFvs were subcloned into an expression vector (pETFlag) and purified from *E. coli* as described (*Materials and Methods*). Their purity was checked by SDS/PAGE and gel filtration and revealed the expected molecular mass of about 30 kDa. Because sLe^x and Le^x share a common biosynthetic pathway and have related carbohydrate structures (35), the fine specificities of the four scFvs were further analyzed by testing the binding to Le^x-BSA by ELISA. It was found that the four scFv antibodies bound sLe^x as well as Le^x (data not shown), therefore sialic acid was not a recognition element for these antibodies. The binding specificities and kinetic parameters of purified scFv antibodies were further determined by using surface plasmon resonance. It was shown that the scFvs bound to immobilized sLe^x-biotin or Le^x-BSA conjugates. Binding to the sensor chips was inhibited by these antigens (data not shown). The dissociation equilibrium constants (K_d) of the scFvs were calculated from association and dissociation rate constants (Table 3). Analysis of the kinetic data revealed that the scFvs, although derived from two different V_H germlines, showed similar affinities for sLe^x and Le^x. The K_d values ranged from 1.1 to 6.2 $\times$ 10^{-7} M. The affinities are the highest that have been observed for antioligosaccharide antibodies directly isolated from phage-antibody libraries. They are also comparable to the affinities of mAbs derived from the murine secondary immune response (11, 15). The scFvs were also analyzed by FACS for binding to human pancreatic adenocarcinoma SW1990 cells to confirm binding to cells overexpressing sLe^x (Fig. 1.). All of the scFv fragments bound to SW1990 cells. With this assay, clone S7 showed the best binding. Given the similar thermodynamic and kinetic behavior of the scFvs in previous assays, the differences in mean fluorescence intensity in binding the cellular epitope likely reflected differences in the presentation of the sLe^x structure and different domains of sLe^x recognized by the scFvs. Indeed, we provided evidence for this hypothesis by performing a two-site BIAcore binding experiment. It was observed in all cases examined that different pairs of scFvs bound concurrently to the sLe^x antigen (Table 4).

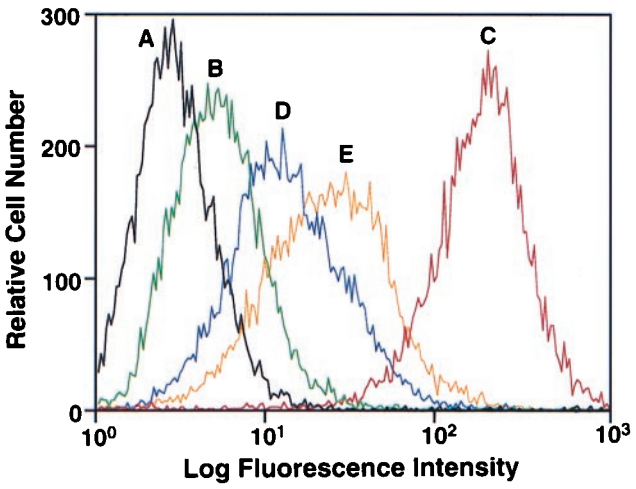

FIG. 1. Binding activity of human scFvs on SW1990 cells measured by using FACS. Cells were incubated in PBS for 1 h at 4°C with (*A*) 1% BSA; negative control; (*B*) S6; (*C*) S7; (*D*) S8; and (*E*) S10. Binding of soluble fragments was detected by adding first anti-Flag M2 murine mAb followed by FITC-conjugated antimurine $F(ab')_2$.

DISCUSSION

Adhesion of cancer cells to vascular endothelium is an important step in hematogenesis and metastasis of cancer. Cell-surface sLe^x has been implicated in tumor-cell binding to endothelial cell-adhesion molecules (selectins) and in cellular extravasation during metastasis (36, 37). High expression of sLe^x, Le^x, or similar carbohydrates in tumors of different organs, correlated with increased metastatic potential of tumor cells and poor patient survival (6, 38). Consequently, antibodies that bind to these carbohydrate antigens are anticipated to have broad application in therapeutic and diagnostic procedures.

The preparation of mAbs against carbohydrate antigens has been achieved by different approaches that included conventional hybridoma technology along with associated humaniza-

Table 4. A BIAcore two-site binding assay of scFvs and sLe^x

	Immobilized scFv*		
Second scFv	S7	S8	S10
S6	+	+	+
S7	−	ND	ND
S8	+	−	+
S10	+	ND	−

*Immobilized scFv was used to trap sLe^x. The second scFv was then tested for binding to the scFv–sLe^x complex. Plus signs (+) indicate scFvs that bound concurrently (typically 100–500 response units) and hence recognized different regions of sLe^x. Minus signs (−) indicate scFvs that did not bind concurrently (typically 0–20 response units). ND, not determined.

tion procedures and affinity selection from recombinant antibody libraries (11, 13–15). Whereas some antibodies from murine sources had good affinity, showed excellent results in animal models, and were engaged in clinical trials, the problem with human antimurine antibodies and other pharmacodynamic effects must be resolved. Passive immunotherapy for cancer could be fully exploited were it possible to have completely human mAbs with high affinity against tumor carbohydrate antigens.

A number of cell-surface antigens, including carbohydrates, mucins, and oncoproteins, were found to elicit a humoral immune response and, in some instances, circulating immune complexes in humans were observed (39–41). The presence of circulating antibodies correlated with antigen overexpression on primary tumors and with advanced-stage disease. Interestingly, in melanoma patients found to have serum antibodies that reacted with their own cell-surface antigens, human mAbs with glycosphingolipid specificity were isolated by using Epstein–Barr virus transformation of PBLs (42, 43). However, these were IgM antibodies undesirable for clinical applications, of low affinity, and difficult to obtain in significant quantities. In general, human mAb technology has lagged far behind that of the well established murine-based technique.

The work of others on antibody isolation from melanoma patients and the known marked accumulation of sLe^x and Le^x antigens in a variety of human cancers served to guide our approach. For these reasons, we chose to construct an antibody library from PBLs of cancer patients in anticipation of readily finding the desired carbohydrate specificities. Notably, the library was constructed to contain only moderate diversity ($\approx 10^8$) that afforded completion in a short period of time and with minimal labor and expense. Yet, the number of positive clones on random selection was extremely high (77%). As a result of the chain shuffling during antibody gene assemblage and the application of a stringent panning protocol, it was possible to select high-affinity anticarbohydrate antibodies directly from the library of phage displaying human scFv antibodies on their surface.

The scFv format was chosen because the expression levels in *E. coli* are typically higher than for Fabs and yield a more efficient antibody display on phage. Furthermore, scFvs should be advantageous for the treatment of solid cancers (27, 28, 44, 45). Because of their small size, these proteins penetrate faster and deeper into tissues and clear more rapidly from the blood than whole IgG or Fabs. Also, the lack of constant regions mitigates against retention by Fc receptors found in most tissues and organs, which further reduces their possible side effects (46, 47).

The aim of our study was to select human antibodies against the sLe^x epitope. However, representation of this epitope with regard to parameters such as conformation, accessible binding determinants, and aggregation states that exist at the tumor surface were not considered and would be difficult to address. Although a direct selection from our library by using cancer cells displaying sLe^x would perhaps be feasible, it is likely that the concomitant selection of irrelevant cell-binding antibodies would necessitate extensive subtraction. Therefore, we used a chemically well defined sLe^x-BSA conjugate as a panning reagent. Indeed, the fact that antibody S7 demonstrated excellent binding to the adenocarcinoma cells suggested that sLe^x and Le^x in the form of BSA conjugates were reasonable mimics of the cell-bound structures. Ultimately, the killing power and therapeutic efficacy of scFv-drug conjugates will depend not only on antibody affinity but also on the differential recognition between cancerous and normal tissue. Whether binding observed in our cellular *in vitro* experiments translates to affinity and specificity against tissue *in vivo* remains to be determined.

The four scFvs examined here had similar kinetic characteristics but showed differences in their binding to tumor cells, which might reflect binding to different determinants. Indeed, together with our other studies, the results from the two-site binding experiments strongly suggested that the scFvs each recognize a specific epitope structure. Clones S6, S8, and S10 were derived from the same germline sequence and probably arose by somatic mutation during the affinity maturation response in the human. It will be of interest to examine other scFvs having a spectrum of affinity for sLe^x to evaluate the role of somatic mutation in generating high-affinity antibodies to this antigen and identifying subtle alterations in carbohydrate recognition. Presumably, there will be an optimum interplay of affinity and specificity for binding the epitope expressed *in vivo* with regard to therapeutic efficacy. Finally, the further examination of the scFvs with respect to binding of antigens expressed in different tumor cell lines will elucidate the primary targets for potential treatment protocols.

Our study demonstrated that high-affinity human antibodies against tumor-associated carbohydrate antigens could be selected from a phage library constructed from the PBLs of various cancer patients. The approach did not depend on immunization procedures or the necessity to repeatedly construct phage antibody libraries. To our knowledge, it is the first time this technique was successfully used for the direct generation of human mAbs against oligosaccharides. Because the mAbs are entirely of human origin and have high affinity, they are expected to be much less immunogenic than murine mAbs and to be efficient in targeting the tumor-cell surface. Consequently, they could prove useful for unparalleled administration as therapeutic reagents in the treatment of cancer.

We gratefully acknowledge the Skaggs Institute for Chemical Biology. We thank Lisa Kerwin for technical assistance.

1. Hounsell, E. F., Young, M. & Davies, M. J. (1997) *Clin. Sci.* **93,** 287–293
2. Kim, Y. S., Gum, J., Jr. & Brockhausen, I. (1996) *Glycoconjugate J.* **13,** 693–707.
3. Bhavanandan, V. P. (1991) *Glycobiology* **1,** 493–503.
4. Hakomori, S. (1989) *Adv. Cancer Res.* **52,** 257–331.
5. Hakomori, S. (1986) *Sci. Am.* **254,** 44–53.
6. Hakomori, S. (1996) *Cancer Res.* **56,** 5309–5318.
7. Muramatsu, T. (1993) *Glycobiology* **3,** 294–296.
8. Hakomori, S. & Zhang, Y. (1997) *Chem. Biol.* **4,** 97–104.
9. Apostolopoulos, V. & McKenzie, I. F. C. (1994) *Crit. Rev. Immunol.* **14,** 293–309.
10. Oettgen, H. F. & Old, L. J. (1991) *Biologic Therapy of Cancer*, eds. DeVita, V., Hellman, S. & Rosenberg, S. A. (Lippincott, Philadelphia).
11. Capurro, M., Bover, L., Portela, P., Livingston, P. & Mordoh, J. (1998) *Cancer Immunol. Immunother.* **45,** 334–339.
12. Miettinen, M. & Karkkainen, P. (1996) *Virchows Arch. B Cell Pathol.* **429,** 213–219.
13. Trail, P. A., Willner, D., Lasch, S. J., Henderson, A. J., Hofstead, S., Casazza, A. M., Firestone, R. A., Hellström, I. & Hellström, K. E. (1993) *Science* **261,** 212–215.
14. Saleh, M. N., Khazaeli, M. B., Wheeler, R. H., Dropcho, E., Liu, T., Urist, M., Miller, D. M., Lawson, S., Dixon, P., Russell, C. H., *et al.* (1992) *Cancer Res.* **52,** 4342–4347.
15. Dinh, Q., Weng, N.-P., Kiso, M., Ishida, H., Hasegawa, A. & Marcus, D. M. (1996) *J. Immunol.* **157,** 732–738.
16. Sakahara, H., Saga, T., Onodera, H., Yao, Z., Nakamoto, Y., Zhang, M., Sato, N., Nakada, H., Yamashina, I., Endo, K., *et al.* (1997) *Jpn. J. Cancer Res. (GANN)* **88,** 895–899.
17. Schroff, R. W., Foon, K. A., Beatty, S. M., Olkham, R. K. & Morgan, A. C., Jr. (1985) *Cancer Res.* **45,** 879–885.
18. Shawler, D. L., Bartholomew, R. M., Smith, L. M. & Dillman, R. O. (1985) *J. Immunol.* **135,** 1530–1535.
19. Daugherty, B. L., DeMartino, J. A., Law, M.-F., Kawka, D. W., Singer, I. I. & Mark, G. E. (1991) *Nucleic Acids Res.* **19,** 2471–2476.
20. Padlan, E. A. (1991) *Mol. Immunol.* **28,** 489–498.
21. Riechmann, L., Clark, M., Waldmann, H. & Winter, G. (1988) *Nature (London)* **332,** 323–327.

22. Co, M. S., Baker, J., Bednarik, K., Janzek, E., Neruda, W., Mayer, P., Plot, R., Stumper, B., Vasquez, M., Queen, C., *et al.* (1996) *Cancer Res.* **56,** 1118–1125.
23. Hand, P. H., Kashmiri, S. V. S. & Schlom, J. (1994) *Cancer* **73,** 1105–1113.
24. Foote, J. & Winter, G. (1992) *J. Mol. Biol.* **224,** 487–499.
25. Zebedee, S. L., Barbas, C. F., III., Hom, Y. L., Caothien, R. H., Graff, R., DeGraw, J., Pyati, J., LaPolla, R., Burton, D. R., Lerner, R. A., *et al.* (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3175–3179.
26. Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C., McCafferty, J., Hodits, R. A., Wilton, J. & Johnson, K. S. (1996) *Nat. Biotechnol.* **14,** 309–314.
27. Jain, R. K. (1990) *Cancer Metastasis Rev.* **9,** 253–266.
28. Milenic, D. E., Yokota, T., Filpula, D. R., Finkelman, M. A., Dodd, S. W., Wood, J. F., Whitlow, M., Snoy, P. & Schlom, J. (1991) *Cancer Res.* **51,** 6363–6371.
29. Welschof, M., Terness, P., Kolbinger, F., Zewe, M., Duebel, S., Doersam, H., Hain, C., Finger, M., Jung, M., Moldenhauer, G., *et al.* (1995) *J. Immunol. Methods* **179,** 203–214.
30. Marks, J. D., Tristem, M., Karpas, A. & Winter, G. (1991) *Eur. J. Immunol.* **21,** 985–991.
31. Gao, C., Lin, C. H., Lo, C. H. L., Mao, S., Wirsching, P., Lerner, R. A. & Janda, K. D. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 11777–11782.
32. Jönsson, U., Fägerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Löfås, S., Persson, B., Roos, H., Rönnberg, I., *et al.* (1991) *BioTechniques* **11,** 620–627.
33. Malmborg, A.-C., Michaëlsson, A., Ohlin, M., Jansson, B. & Borrebaeck, C. A. K. (1992) *Scand. J. Immunol.* **35,** 643–650.
34. Ho, J. J. L., Siddiki, B. & Kim, Y. S. (1995) *Cancer Res.* **55,** 3659–3663.
35. Stroud, M. R., Levery, S. B., Maartensson, S., Salyan, M. E. K., Clausen, H. & Hakomori, S. (1994) *Biochemistry* **33,** 10672–10680.
36. Martin-Satue, M., Marrugat, R., Cancelas, J. A. & Blanco, J. (1998) *Cancer Res.* **58,** 1544–1550.
37. Hiraiwa, N., Dohi, T., Kawakami-Kimura, N., Yumen, M., Ohmori, K., Maeda, M. & Kannagi, R. (1996) *J. Biol. Chem.* **271,** 31556–31561.
38. Mayer, B., Funke, I. & Johnson, J. P. (1996) *Gastroenterology* **111,** 1433–1446.
39. Gourevitch, M. M., von Mensdorff-Pouilly, S., Litvinov, S. V., Kenemans, P., van Kamp, G. J., Verstraeten, A. A. & Hilgers, J. (1995) *Br. J. Cancer* **72,** 934–938.
40. Lloyd, K. O. (1991) *Semin. Cancer Biol.* **2,** 421–431.
41. Singhal, A. K., Singhal, M. C., Nudelman, E., Hakomori, S., Balint, J. P., Grant, C. K. & Snyder, H. W., Jr. (1987) *Cancer Res.* **47,** 5566–5571.
42. Lloyd, K. O. (1993) *Ann. N. Y. Acad. Sci.* **690,** 50–58.
43. Yamaguchi, H., Furukawa, K., Fortunato, S. R., Livingston, P. O., Lloyd, K. O., Oettgen, H. F., Old, L. J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3333–3337.
44. Mayer, B., Funke, I. & Johnson, J. P. (1996) *Gastroenterology* **111,** 1433–1446.
45. Deng, S.-J., MacKenzie, C. R., Hirama, T., Brousseau, R., Lowary, T. L., Young, N. M., Bundle, D. R. & Narang, S. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 4992–4996.
46. Yamaguchi, A., Ding, K., Maehara, M., Goi, T. & Nakagawara, G. (1998) *Oncology* **55,** 357–362.
47. Nakashio, T., Narita, T., Sato, M., Akiyama, S., Kasai, Y., Fujiwara, M., Ito, K., Takagi, H. & Kannagi, R. (1997) *Anticancer Res.* **17,** 293–299.